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# Molecular phylogeography of the polyploid complex *Bupleurum ranunculoides* s.l. L. (Apiaceae)

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**Abstract**

The alpine plant *Bupleurum ranunculoides* L. s.l. (Apiaceae) is widely distributed in the European mountains, from the Cantabric range to the Carpathians, passing through the Pyrenees and the Alps. Both the dispersal and the survival of *B. ranunculoides* are strongly challenged by adequate habitats composed of calcareous substrates, which might have reinforced the tough constrains endured during the Pleistocene glaciations. According to the extant literature, this species comprises different cytotypes ranging from diploids to hexaploids that demonstrate a clear pseudo-vicariance: whereas the diploid forms are distributed in the Southern part of the distribution area, the polyploid forms expand throughout the species Northern edge. In this study, we aimed to disentangle the biogeographic history of *B. ranunculoides* by analyzing the spatial genetic structure of this species using an extensive sampling comprising 53 populations distributed throughout its whole range. Using flow cytometry and manual chromosome counting, we were able to provide some supplemental information regarding the distribution of the diploid, tetraploid and hexaploid cytotypes, with hexaploid cytotypes restricted to the populations North to the Alps. Using AFLP genome fingerprinting and ITS sequencing, we could highlight a relatively deep split between two lineages distributed North vs. South to the Alps. More precisely, five different genetic clusters, two in the Northern and three in the Southern clades were highlighted based on the AFLP data. We suggest that the three genetic clusters from the Southern Alps have survived glaciations in Iberia, Italy, and Balkan refugia, respectively. Regarding the two lineages in the Northern Alps, our data might suggest *in situ* survival in a peripheral refugium during the last ice age. While both the Northern and Southern lineages have experienced polyploidization processes, only the former encompasses hexaploid lineages. Our study highlights the recent evolutionary history of *B. ranunculoides* and demonstrates that subspecific treatments are not readily applicable to a taxon with intricate and parallel polyploidization events.

***Keywords:*** phylogeography, polyploidy, AFLP, ITS, Alps, peripheral refugia, Pleistocene climatic oscillations.

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**Labhardt A., Schmid S., Buerki S., Espindola A., Ronikier M., Küpfer P., Arrigo N. & Alvarez N., 2020. Phylogénie moléculaire du complexe polyploïde *Bupleurum ranunculoides* s.l. L. (Apiaceae). *Bulletin de la Société Vaudoise des Sciences Naturelles* 99: 83-106.**

**RÉSUMÉ**

La plante alpine *Bupleurum ranunculoides* L. s.l. (Apiaceae) est largement répandue dans les montagnes européennes, de la chaîne Cantabrique aux Carpates, en passant par les Pyrénées et les Alpes. Tant la dispersion que la survie de *B. ranunculoides* sont fortement influencées par des habitats adéquats composés de substrats calcaires, qui pourraient avoir renforcé les fortes contraintes subies pendant les glaciations du Pléistocène. Selon la littérature existante, cette espèce comprend différents cytotypes allant des diploïdes aux hexaploïdes qui présentent une pseudo-vicariance évidente: alors que les formes diploïdes sont réparties dans la partie sud de l'aire de répartition, les formes polyploïdes s'étendent sur toute la bordure nord de l'espèce. Dans cette étude, nous avons cherché à démêler l'histoire biogéographique de *B. ranunculoides* en analysant la structure génétique spatiale de cette espèce à l'aide d'un vaste échantillonnage comprenant 53 populations réparties dans toute son aire de répartition. En utilisant la cytométrie de flux et le comptage manuel des chromosomes, nous avons pu fournir quelques informations supplémentaires concernant la distribution des cytotypes diploïdes, tétraploïdes et hexaploïdes, montrant que les cytotypes hexaploïdes sont limités aux populations situées au Nord des Alpes. En appliquant des protocoles moléculaires de polymorphisme de longueur de fragments amplifiés (AFLP) et de séquençage de la région nucléaire ITS, nous avons pu mettre en évidence une divergence relativement profonde entre deux lignées distribuées au Nord et au Sud des Alpes. Plus précisément, cinq groupes génétiques différents, deux dans le groupe Nord et trois dans le groupe Sud ont été mis en évidence sur la base des données AFLP. Nous suggérons que les trois groupes génétiques des Alpes du Sud ont survécu aux glaciations dans les refuges se trouvant dans la péninsule Ibérique, l'Italie et les Balkans. En ce qui concerne les deux lignées dans les Alpes du Nord, nos données pourraient suggérer une survie *in situ* dans un refuge périphérique pendant la dernière période glaciaire. Alors que les lignées Nord et Sud ont toutes deux connus des processus de polyploïdisation, seule la première englobe des lignées hexaploïdes. Notre étude met en évidence l'histoire récente de l'évolution de *B. ranunculoides* et démontre que les traitements systématiques infraspécifiques ne sont pas facilement applicables à un taxon présentant des événements de polyploïdisation complexes et parallèles.

***Mots-clés:*** phylogéographie, polyploïdie, AFLP, ITS, Alpes, refuge périphérique, oscillations climatiques du Pleistocène.

## Introduction

In Europe, mountain ranges constitute a discontinuous system creating relatively well segregated geographic and ecologic islands for alpine and subalpine plant species (Ozenda 1985). During the Pleistocene climatic oscillations and especially through the last glacial maximum (LGM), the mountain ranges of the Cantabric chain, the Pyrenees, Alps, Carpathians and Caucasus were all covered by large ice sheets, while between them and the Northern ice-cap, the plains of Europe were covered by tundra and cold steppe vegetation (Hewitt 1996). In this context, most taxa were restricted to the potential refugial areas located in the South of Europe, for instance the Balkans, Italy and the Iberian Peninsula including Southern France, plus an extra Eastern Europe refugium in the Carpathians (Taberlet 1998), whose ice cover was much lower than in other mountainous ranges. At that time, currently isolated popula-

tions of alpine species could have been interconnected because of the downshift of the vegetation belt, creating gene exchange possibility and species migration (Ozenda 1985). During the subsequent warming, post-glacial colonization routes were diverse but a general trend has been drawn. Most taxa followed the recolonization route from the Iberian, Balkan and Carpathians refugia to the North, whereas the huge barrier of the Alps blocked most Italian populations. After postglacial expansion, suture-zones were produced by the meeting of major diverged genomes, as for instance in the Pyrenees and Northern Alps (Taberlet 1998; Hewitt 2000; Comes & Kadereit 2003; Sanz *et al.* 2014). Range expansions and contractions due to climatic oscillations produced drastic genetic consequences at the intraspecific level. As predicted by models and verified by different studies, it has not only eliminated haplotypes present in glaciated areas, but has also created a much higher intraspecific diversity in Southern refugia areas than in postglacial colonized regions (Küpfer 1981; Taberlet 1998; Scotti-Saintagne *et al.* 2019). Thus, isolation in the South has been ideal for intraspecific differentiation, and the distribution of polymorphism in Northern regions has been dictated by the colonization-routes used from the refugia (Taberlet 1998). Mountains have often been seen as major barriers blocking the advancement of taxa during their postglacial expansion. Nevertheless, several studies have shown that perialpine refugia and central nunataks were used by alpine plants to survive the LGM (Lohse *et al.* 2011; Schneeweiss & Schönswetter 2011; Schönswetter & Schneeweiss 2019). The diverse topographical patterns found in alpine areas resulted in a variety of microclimates, which could have provided suitable habitats during both warm and cold periods (Taberlet & Cheddadi 2002). Presently, much effort is being done to address the location of specific refugia in the mountains surrounding the main massifs or in nunataks, and also to interpret postglacial recolonization processes. Thus, based upon floristic and ecological biogeographic literature, Stehlik (2000) drew a map of potential peripheral refugia in the Alps and showed that many of them – mainly siliceous – were not only situated in the Southern Alps, but also at the edge of Northern calcareous Alps, and towards the East regarding calcicolous taxa. Afterwards, based upon molecular phylogeographic studies, Schönswetter and colleagues (2005) established the location of different potential refugia along the immediate surrounding of Southern Alps, for both calcareous and siliceous plants ecological requirements, which were confirmed as partly driving spatial genetic structures (Alvarez *et al.* 2009, Thiel-Egenter *et al.* 2011). Nowadays, a large number of studies have been done on specific alpine plants using new molecular techniques, allowing improvement in the knowledge of plant phylogeography (e.g. Casazza *et al.* 2016; Maccagni *et al.* 2017; Rogivue *et al.* 2018)

## Evolution of polyploidy in the Alpine context

In a context of postglacial colonization and according to the numerous strategies developed by plants to adapt to environmental changes, polyploidy plays an important role by increasing recolonization chances (Küpfer 1974). The origin of polyploids, as well as the conditions that favoured the establishment and persistence of recently formed polyploids are still not completely understood (Rehman 2017). However, more than 50 years ago, Favarger (1967) already observed that natural young polyploids often showed a periglacial origin. Indeed, the widely accepted Stebbins's "secondary contact hypothesis" (1984) suggests that range shifts caused by climate oscillations might have promoted hybridization between previously separated popula-

tions, which may have survived in nunataks or in peripheral refugia. As a consequence, these populations may have accumulated a large genetic differentiation and the hybridization of those intra-specific lineages is often accompanied or followed by polyploidy and introgression (Stebbins 1984). In the case of polyploidy, the further combination of genetic information proceeding from two diverging parental lineages may provide polyploids a higher physiological and ecological flexibility promoted by high level of heterozygosity (Küpfer 1974; Stebbins 1984). Thus, neopolyploids would have better adaptive aptitudes and greater colonizing abilities to new environments compared to their progenitors (Petit & Thompson 1999). As suggested by Soltis *et al.* (2004), the coexistence of the new-formed race with its diploid ancestor is promoted by habitat differentiation immediately following the polyploidization events (Arrigo *et al.* 2016). As a consequence, polyploid plants are often found on post-glaciated areas. Commonly, it is accepted that polyploidy can have monospecific or para/polyspecific origins producing respectively autopolyploids and allopolyploids (Ramsey & Schemske 2002). At the intraspecific level, the definitions of auto- and allo-ploidy can be expanded (*i. e.*, "homozygous autopolyploidy" if the parental genotypes are identical or "segmental allopolyploidy" if the parental genotypes proceed from different lineages; Bretagnolle *et al.* 1998). Interestingly, the reproductive mode of polyploid individuals can experience three potential consequences of the evolution to a genome-wide multiplication of chromosome number. First, a sensitive decrease of male and female fertilities often follows polyploidization. Second, an increase of the frequency of autogamy has been verified in different studies, although the mechanisms leading to self-incompatibility are still not well known. Lastly, polyploidy might favour the occurrence and establishment of apomictic lineages (asexual reproduction by seeds) (Bretagnolle *et al.* 1998).

Compared to other polyploid species, biogeographic studies of polyploid alpine plants are less frequent (Burnier *et al.* 2009; Fernández Prieto *et al.* 2017). Thus, based on Küpfer's (1974) studies and using modern molecular methods, this study aims to revisit the biogeographic history of the polyploid complex *Bupleurum ranunculoides* s.l. L. (Apiaceae). *Bupleurum ranunculoides* s.l. L. is a long-lived orophytic species widespread throughout the mountains of central Europe, Cantabric range, Pyrenees, Central Massif, Alps, Apennines, Jura, and the Carpathians Mountains. It grows almost exclusively on calcareous bedrock in open grassland communities, often on grassy edges, rocky outcrops or stabilised scree fields, always where it can get full sunlight and where it does not have to support a long snow cover. Various subspecies were described during the past decades. The subspecies *Bupleurum ranunculoides ranunculoides* L. is composed of diploid, tetraploid and hexaploid forms (Cauwet 1979). It is the most alpine form and does not exceed 40 cm high, with wide leaves and bracts. It is distributed in the whole species range (figure 1A). The subspecies *Bupleurum ranunculoides caricinum* (DC.) Arcangeli is only composed of diploids and is more restricted to the Southern Alps. Morphologically, it has smaller and more elongated leaves and bracts, and it can grow up to 60 cm high (figure 1B). Finally, the subspecies *Bupleurum ranunculoides telonense* (Gren. ex Timb.-Lagr.) Bonnier is composed of diploids and tetraploids (Cauwet 1979); it is the Mediterranean form confined to Southern France and Spain, which also has small elongated leaves and bracts. The basal leaves are also longitudinally enrolled, the stem has many ramifications and it can grow up to 80 cm high (figure 1C). Various levels of ploidy have already been described in previous studies with diploid populations (2n = 2x = 14) pre-

sent in the Southern part of the range and in the Tatras, tetraploids (2n = 4x = 28) present in the Pyrenees, Central massif and the Western Alps including Southern Jura, and hexaploids (2n = 6x = 42) confined to the Northern Alps and Northern Jura plus in the Cantabric range (Küpfer 1974). In his work, Küpfer (1974) demonstrated a Southern-Northern pseudo-vicariance for *B. ranunculoides* s.l. and a polytopic origin of the hexaploid in the Cantabria chain and in the Alps. A unique population of triploid (2n = 3x = 21) had been found in the Oriental part of Pyrenees (Cauwet 1970). Cauwet (1979) showed that in the oriental Pyrenees, the north-western limit of the distributions of diploid populations was exactly correlated with the front of quaternary ice sheet and that, tetraploids were often found on post-glacial and neo-glacial reliefs. Because of the coexistence of the diploid ancestor and the polyploid descendant in this narrow area, *B. ranunculoides* has been considered as a neopolyploid in the Pyrenees (Cauwet 1979).

The taxonomy of the *Bupleurum ranunculoides* s.l. group is complex because of a high level of polymorphisms in phenotypic traits. Its broad distribution has caused a subspecies splitting into a multitude of taxa. Cauwet (1979) listed about 100 subspecies, varieties and morphotypes but she did not validate them after comparing a large set of characters in the plant's morphology, anatomy, phyto-chemistry, palynology, caryology as well as by performing phytodermic analysis. In conclusion, she considered that not enough conspicuous relations could be shown to establish infraspecific taxonomic ranks for *B. ranunculoides* (Cauwet 1979). Those considerations have not been recognized by the scientific community since the three subspecies mentioned above still exist according to the National Data and Information Center on the Swiss Flora (Info Flora) and to the Index Synonymique de la Flore de France.

The aims of this study are thus to (1) clarify the ploidy distribution of *B. ranunculoides* s.l. throughout almost the whole geographical range with a special focus on the Eastern Alps, which have not been previously analysed; (2) describe intraspecific genetic structure by identifying lineages; (3) propose a new molecular phylogeographic hypothesis for the species complex.

## Materials and methods

### Sampling

We analysed a total of 257 individuals of *Bupleurum ranunculoides* s.l. L. sampled from 53 populations covering the whole geographic range of the species, with the exception of the Cantabric region and the Apennines (table 1). We also included individuals from five other species of *Bupleurum* in order to confirm the paraphyly of the species addressed by Neves and Watson 2004. Determination of specimens was done in the field according to Aeschimann and Burdet (1994) and Bonnier (1934). However, since the level of polymorphism was larger in the field than expected in the literature, some samples were assigned to a subspecies using the "confer" latin abbreviation (cf.). In each population, young leaves of 5 to 8 individuals were collected in the field and immediately dried into silica gel (Chase & Hills 1991). One or two individuals per population were also transplanted at the Botanical Garden of the University and City of Neuchâtel for ploidy level determination. Despite the number of transplanted specimens is rather low, extrapolation to the ploidy level of populations to which they belong is considered consistent (Küpfer, pers. obs.).

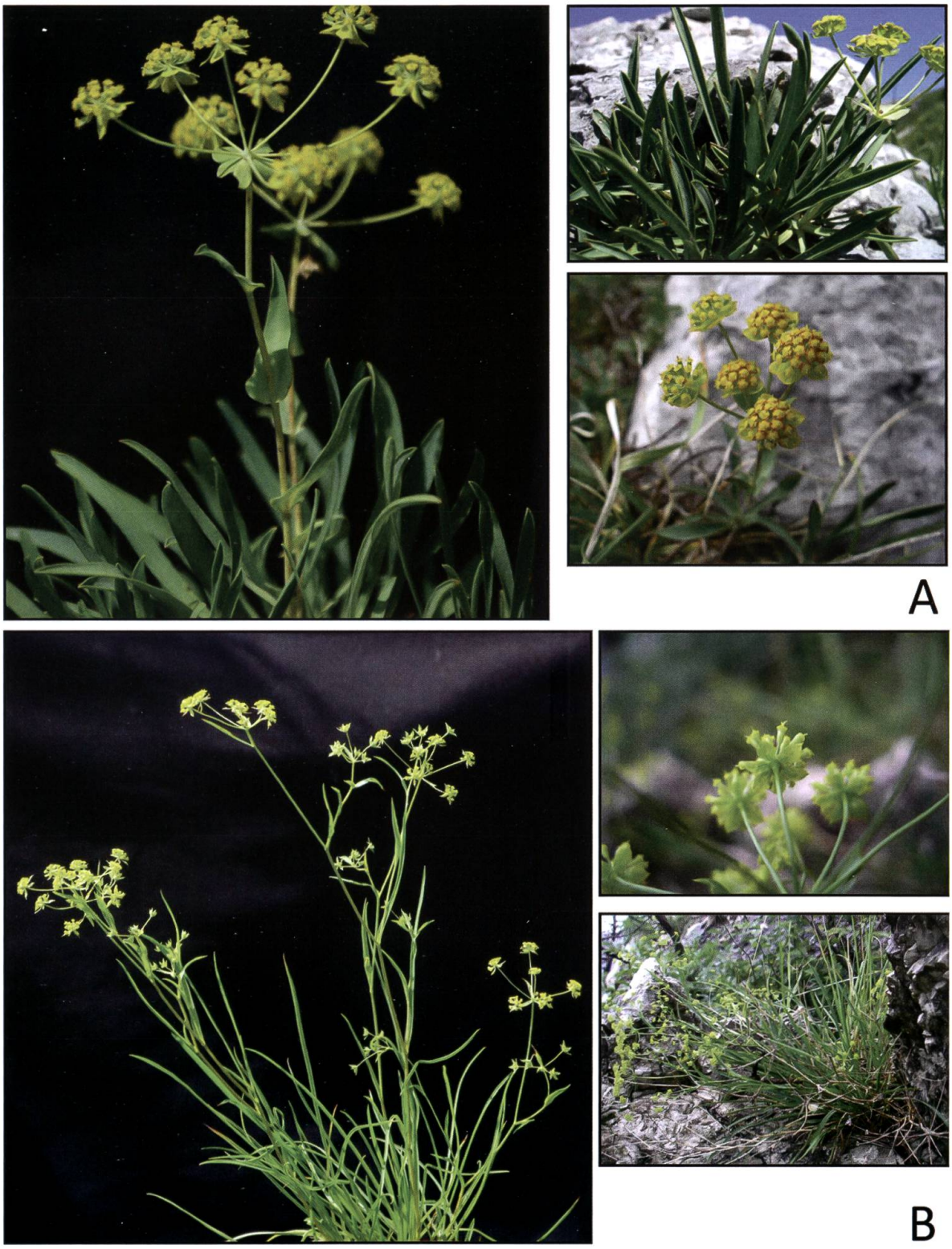



Figure 1. Picture of the three *Bupleurum ranunculoides* subspecies. A) *Bupleurum ranunculoides* subsp. *ranunculoides* L. Individuals from Le Suchet, Vaud ; B) *Bupleurum ranunculoides* subsp. *caricinum* (DC) Arcangeli. Individuals from Amaro, Italy ; C) (see next page) *Bupleurum ranunculoides* subsp. *telonense* (Gren. ex Timb.-Larg.) Bonnier. Individual from the Carpathians (left) and individual from Mont Faron, Toulon (center and right).

C

## Ploidy level determination

Determination of ploidy level was done by chromosomal counts on the transplanted plants. Root tips were pre-treated in water saturated in bromonaphtalen during 3 hours rinsed and fixed in a solution of methanol-acetic acid (3:1) for several days. Colouration was done by staining root tips into acetic carmine for 2 hours. The solution was heated up to boiling for 2-3 minutes and then, cooled down by adding 45 % acetic acid. Afterwards, root tips were squashed and observed with microscope. Every count has been verified by DNA flow cytometry at the University of Fribourg. The nuclear suspensions were prepared by chopping 200 mg (approx. 0.5 $cm^2$) of fresh leaf tissue with a razor blade during 30 seconds in glass Petri dish containing a total of 5 ml of extraction buffer. After 90 seconds of incubation the suspension was filtered using CellTrics® (filters made from monofil nylon material) for isolation of cells and nuclei from cells debris and aggregates before adding 2 ml of staining buffer. After another incubation of 30 minutes, the samples were analysed in the flow cytometer (Partec PA Ploidy analyser). Ploidy level was mapped geographically using ArcGIS 9.

## DNA Extraction

Extraction of DNA was done on dried leaves using the QIAGEN DNeasy plant kit (Qiagen, Hilden, Germany) following the manufacturer protocol, including a 1 % RNase treatment during cell lysis. The extracted DNA was suspended in elution buffer and stored at -20 °C in the Laboratory of the Evolutionary Botany of Neuchâtel. Its quality was checked on a 1 % agarose gel stained with ethidium bromide.

## AFLP fingerprinting

The AFLP procedure followed the general protocol described in Vos *et al.* (1995) with minor modifications and using non-radioactive fluorescent dye-labeled primers (PE Biosystems). Genomic DNA was digested with the restriction enzymes *Eco*RI (Promega) and *Mse*I (New England Biolabs) and double-stranded adapters were ligated to the digested DNA. Preselective amplification was performed using primer pairs with a single selective nucleotide, *Eco*RI-A and *Mse*I-C. Selective amplifications were performed with the two primer combinations *Eco*RI-ACC/*Mse*I-CTG and *Eco*RI-ACA/*Mse*I-CTA. Selective amplification products were run by Macrogen Inc. (Seoul, South Korea) in a denaturing polyacrilamide gel with an internal size standard (Dye set DS-30 & Filter set D: 6-FAM, HEX, NED, ROX/Standard; 400HD) on an automated DNA sequencer (ABI 377).

Raw AFLP data was collected and sized using the Genescan® 3.7 Analysis Software (PE Applied Biosystems). The AFLP profiles were scored using the software Genographer 1.6.0 (Benham *et al.* 1999). Only easily scorable and unambiguous fragments in the range 50 to 400 bp in length were recorded manually and coded as presence (1) or absence (0). Scoring was done separately for the two primers combination, and then the two matrices were assembled. In a second step, we performed an automatic scoring of the raw chromatograms by using RawGeno 1.0 (Arrigo *et al.* 2009) in order to build an extremely large matrix (~1000 markers per primer pair) and to test the robustness of the manual scoring in the resulting patterns.

## Nuclear ITS region sequencing

The ITS nuclear markers (ITS1, 5.8S, ITS2) were amplified and sequenced for a representative subset of the sampling composed of 84 *Bupleurum ranunculoides* s.l. Primers are described in White *et al.* (1990). Amplification of selected regions was done in a 25 µl reaction mixture containing 5 µl PCR buffer (5X), 5 µl Qsolution, 1.1 µl $MgCl_2$ (25 mm), 0.5 µl dNTPs, 0.5 µl primers (10 nM), 0.2 µl GoTaq polymerase (5 u/µl) and 10.2 µl $ddH_2O$. Initial template denaturation was programmed for 2 minutes at 95 °C, followed by 28 cycles at 95 °C for 45 seconds, 52 °C for 45 seconds, 72 °C for 1 minute, plus a final extension of 10 minutes at 72 °C. Purification and sequencing were performed by Macrogen, Inc (Seoul, South Korea).

## Spatial genetic structure and phylogenetic analysis

### BAYESIAN analysis

The 268 individuals were analysed through Bayesian inference clustering using the programme STRUCTURE 2.2, which clusters individuals based on their multilocus genotype using a Markov Chain Monte Carlo (MCMC) algorithm and a within-clusters Hardy-Weinberg equilibrium assumption (Pritchard *et al.* 2000; Falush *et al.* 2007). The admixture model was used assuming independence of allele frequencies among populations, and parameterization was set up adequately for dominant markers (*i. e.* adapted for AFLP data sets) and for copy number variation, as the data included polyploidy cytotypes. The *K* value (a user-defined number of clusters) was set from 1 to 9. Five independent runs were carried out for each value of *K*, with a burn-in period of 200,000 generations and followed by a 1,000,000 Monte Carlo Markov Chains (MCMC) generations. For each *K* value, only the run yielding the best likelihood was considered. The best *K* value was selected according to the behaviour of the like-

lihood function. Assignment probabilities of each population was mapped using ArcGIS 9. At the individual level, assignment to a cluster was defined according to the majority-rule criterion (an individual was assigned to a given cluster assuming that its assignment probability was above 0.5).

### Principal coordinate analyses (PCoA)

AFLP data was explored by principal coordinate analyses (PCoA; Krzanowski 1990) using the Vegan R package (Oksanen *et al.* 2013) in *R* 2.1.0. This analysis was conducted using the Jaccard's similarity coefficient, and the first two axes were plotted in two-dimensional graphs. Results of STRUCTURE and ploidy level were mapped afterwards.

### AFLP phylogenetic reconstruction

The AFLP matrix was analysed using the Nei-Li distance criterion and a phylogeny based on the resulting distance matrix was constructed using a neighbour-joining algorithm as implemented in PHILIP 3.67 (Felsenstein 2007). In order to avoid long branches attraction, and because only very few loci were shared between the focal and outgroup species, outgroup species were discarded from the analysis. The Nei-Li distance measures the probability that a band being amplified in one sample is also amplified in another sample. It is defined as $2n_{xy}/n_x+n_y$, with $n_x$ and $n_y$ being the total of fragments in accessions x and y respectively, and $n_{xy}$ the number of fragments shared between the two accessions. Another among-clusters distance matrix based on $F_{ST}$ values was computed using STRUCTURE 2.2. We reconstructed the hierarchical relations among clusters using the neighbour-joining algorithm implemented in PHILYP 3.67 (Felsenstein 2007). Finally, we calculated an index of genetic diversity following to the IntraBioDiv project protocol (Alvarez *et al.* 2009).

### ITS sequence analysis

ChromasPro 1.34 was used to assemble complementary strands and check software base-calling. The ITS region was initially aligned using Clustal W (Larkin *et al.* 2007). Because of the low level of polymorphism retrieved, ITS alleles were directly plotted on a geographical map.

## Results

### Ploidy level determination

The analysis of the ploidy levels showed three different cytotypes: the diploids (2n = 2x = 14), the tetraploids (2n = 2x = 28) and the hexaploids (2n = 6x = 42). Results from chromosomal counts and DNA flow cytometry were fully congruent. The ploidy levels are indicated in table 1 and mapped geographically on figure 2. According to our results, all populations seem only composed by one single cytotype, even though the low number of specimens analysed could bias this result. Overall, spatial structuring of the ploidy level argues in favour of one single ploidy level per population. Generally, diploid specimens are distributed in the Southern Alps, the Oromediterranean part of France and in the Tatra (most Eastern population). Tetraploids occurred in the Pyrenees, Central massif, North-Western Alps, Southern Jura and Eastern Alps. Hexaploid cytotypes were surrounded by tetroploid populations and were restricted to the Jura and the Northern Alps from Le Suchet to Aggenstein.

Table 1. Details on the samples analysed in this study: Population number, Acronym, Ind = number of individuals analysed, Species according to morphology and distribution, Country, Region, Locality, Coordinates (E/N), Ploidy level (2n) of 53 populations of *Bupleurum ranunculoides* s.l. L. and 5 populations of *Bupleurum* sp. Vouchers specimens of every population are deposited at the herbarium of the University of Neuchâtel (NEU). Pop with * have less than 85% assigned to only one cluster in Bayesian structuration.

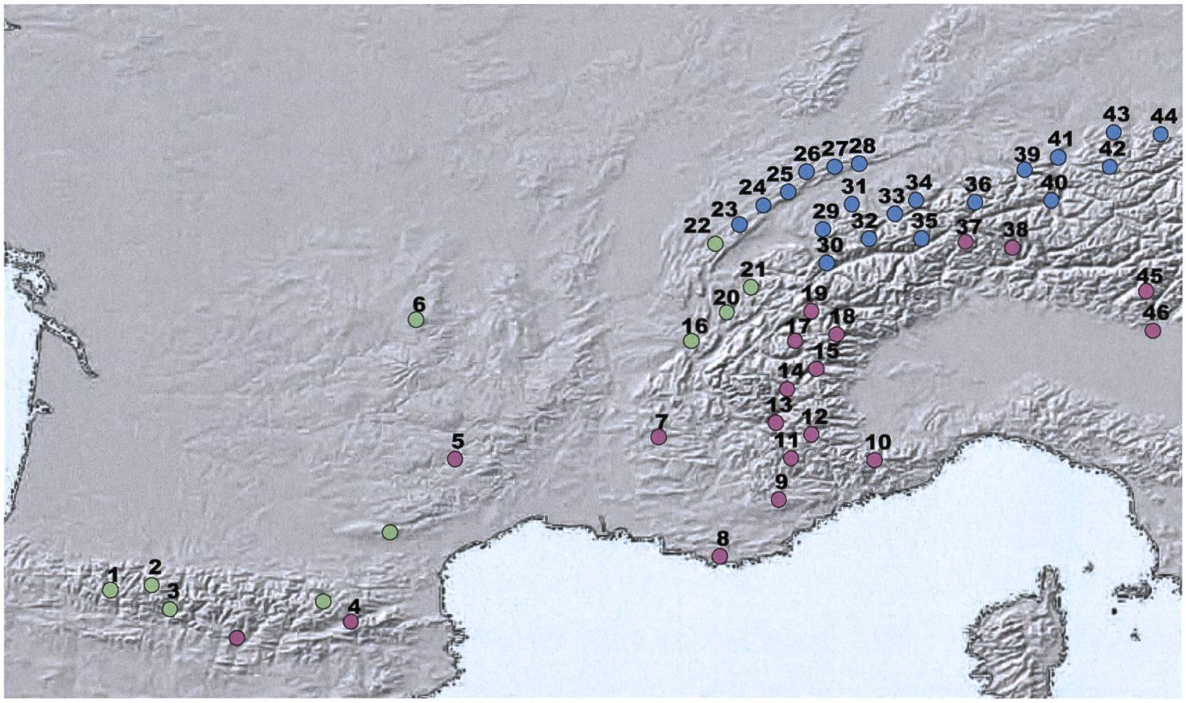
| Pop | Acronym | Ind. | Species | Country | Region | Locality | Coordinates(E/N) | | Ploidy level (2n) | Alt. |
|---|---|---|---|---|---|---|---|---|---|---|
| **Ingroup** | | | | | | | | | | |
| 1* | PORTE | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Pyrénées-Atlantiques | Col du Pourtalet | 000°25'04 | 42°48'11 | 28 (4x) | 1782 |
| 2 | SAUG | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Hautes-Pyrénées | Pâturage du Saugué, entre Gèdre et Gavarnie | 000°00'24 | 42°47'57 | 28 (4x) | 1542 |
| 3* | CBIEL | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Hautes-Pyrénées | Montagne du Campbiel | 000°03'15 | 42°47'08 | 28 (4x) | 1705 |
| 4* | EYNE | 5 | *Bupleurum ranunculoides* subsp. cf. *ranunculoides* L. | France | Pyrénées Orientales | Entrée de la Vallée d'Eyne | 002°05'08 | 42°27'59 | 14 (2x) | 1638 |
| 5 | CAUS | 5 | *Bupleurum ranunculoides* subsp. *telonense* (Gren. ex Timb.-Larg.) Bonnier | France | Aveyron | Causse noir entre le Sonnac et la route de Longuiers | 003°10'48 | 44°09'42 | 14 (2x) | 800 |
| 6 | BADO | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Puy-de-Dôme | Banne d'Ordanche | 002°46'19 | 45°36'40 | 28 (4x) | 1495 |
| 7 | MONTR | 5 | *Bupleurum ranunculoides* subsp. cf. *telonense* (Gren. ex Timb.-Larg.) Bonnier | France | Drome | Montréal-les-Sources à Serre Chapeau | 005°18'18 | 44°23'13 | 14 (2x) | 1150 |
| 8 | FARON | 5 | *Bupleurum ranunculoides* subsp. *telonense* (Gren. ex Timb.-Larg.) Bonnier | France | Var | Mont Faron, Toulon | 005°56'45 | 43°09'03 | 14 (2x) | 505 |
| 9 | BRUI | 5 | *Bupleurum ranunculoides* subsp. *telonense* (Gren. ex Timb.-Larg.) Bonnier | France | Alpes Maritimes | Montagne du petit Brouis | 006°33'30 | 43°44'34 | 14 (2x) | 1200 |
| 10 | TENDE | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Italy | Piemonte | Col de Tende | 007°33'44 | 44°08'59 | 14 (2x) | 1850 |
| 11 | CHPS | 3 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Alpes Maritimes | Col des Champs | 006°41'31 | 44°10'21 | 14 (2x) | 2148 |
| 12* | MADA | 5 | *Bupleurum ranunculoides* subsp. cf. *ranunculoides* L. | France | Alpes de Haute Provence | Colle della Maddallena, Col de Larche | 006°53'57 | 44°25'20 | 14 (2x) | 1970 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | VARS | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Hautes Alpes | Col de Vars | 006°02'04 | 44°32'25 | 14 (2x) | | 2100 |
| 14 | BRIAN | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | France | Hautes Alpes | Briançon | 006°39'15 | 44°53'22 | 14 (2x) | | 1600 |
| 15 | BESS | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | France | Savoie | Col de la Madeleine entre Bessans et Lans | 006°57'04 | 45°18'06 | 14 (2x) | | 1688 |
| 16* | RUCH | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Chartreuse | Petit Som depuis le col de la Ruchère | 005°48'19 | 45°23'12 | 14 (2x) | | 1630 |
| 17 | BOCHO | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | France | Savoie | Mont Bochor au-dessus de Pralognan-la-Vanoise | 006°44'02 | 45°23'10 | 14 (2x) | | 1950 |
| 18 | ISERA | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Savoie | Pont St Charles après Val d'Isère | 007°02'18 | 45°27'18 | 14 (2x) | | 2050 |
| 19 | PSTB | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Italy | Savoie | Col du Petit St Bernard | 006°54'06 | 45°41'50 | 14 (2x) | | 2038 |
| 20 | TRELO | 2 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Savoie | Le Trélod | 006°11'28 | 45°41'10 | | 28 (4x) | 2100 |
| 21* | ROC | 4 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | France | Haute Savoie | Roc des Bœufs, sur Chapelle-St-Maurice | 006°10'02 | 45°45'36 | | 28 (4x) | 1710 |
| 22 | DOLE | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Vaud | La Dôle | 006°06'15 | 46°25'30 | | 28 (4x) | 1470 |
| 23 | SUCH | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Vaud | Le Suchet | 006°27'57 | 46°46'22 | | 42 (6x) | 1559 |
| 24 | CHAS | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Vaud | Le Chasseron | 006°32'07 | 46°50'51 | | 42 (6x) | 1535 |
| 25* | CRDV | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Neuchâtel | Creux du Van | 006°43'13 | 46°56'13 | | 42 (6x) | 1405 |
| 26 | CRO | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Neuchâtel | Roche aux Crôs | 006°51'38 | 47°04'11 | | 42 (6x) | 1329 |
| 27* | CBEG | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Bern | Combe Grède | 007°02'24 | 47°08'08 | | 42 (6x) | 1677 |
| 28 | OGRB | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Solothurn | Obergrenchenberge | 007°24'06 | 47°13'48 | | 42 (6x) | 1365 |

Table 1. (following).

| Pop | Acronym | Ind. | Species | Country | Region | Locality | Coordinates(E/N) | | Ploidy level (2n) | Alt. |
|---|---|---|---|---|---|---|---|---|---|---|
| 29 | MOLET | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Fribourg | Le Moléson | 007°01'02 | 46°32'54 | 42 (6x) | 1999 |
| 30 | DOR | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Vaud | Mont D'or | 007°03'36 | 46°23'18 | 42 (6x) | 2177 |
| 31* | BADH | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Bern | Bäderhorn au-dessus du Jaunpass | 007°19'25 | 46°36'38 | 42 (6x) | 1907 |
| 32 | HAN | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Bern | Hahnenmoospass entre Adelboden et Lenk | 007°29'58 | 46°27'00 | 42 (6x) | 1999 |
| 33 | NIDR | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Bern | Niederhorn sur les Beatenberg | 007°46'23 | 46°42'38 | 42 (6x) | 1949 |
| 34* | STRIK | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Luzern | Strick, sommet N-E des Schrattenflue | 007°59'10 | 46°51'13 | 42 (6x) | 1924 |
| 35 | BAREG | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Bern | Bäregg, au-dessus de Grindelwald | 008°03'17 | 46°36'35 | 42 (6x) | 1423 |
| 36 | BOGLI | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Uri | Bogli | 008°37'09 | 46°49'30 | 42 (6x) | 1449 |
| 37 | CAMPO | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | Switzerland | Ticino | Campo, au-dessus de St Carlo | 008°31'07 | 46°25'14 | 14 (2x) | 1368 |
| 38 | SARO | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | Switzerland | Ticino | Sasso Rosso, Mte Bré | 009°00'36 | 46°21'23 | 14 (2x) | 1270 |
| 39* | MATT | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | St. Gallen | Mattstock depuis Amden | 009°08'24 | 47°10'06 | 42 (6x) | 1255 |
| 40* | GONZ | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | St. Gallen | Gonzen | 009°25'08 | 47°04'37 | 42 (6x) | 1838 |
| 41 | APP | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Switzerland | Appenzell | Kamor, Appenzell | 009°29'27 | 47°17'44 | 42 (6x) | 1604 |
| 42 | HIRCH | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Austria | Vorarlberg | Schönenbach / Hirschberg | 010°01'48 | 47°22'28 | 42 (6x) | 1820 |
| 43 | HGR | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Germany | Allgäu, Bade-Wurtemberg | Hochgrat | 010°04'25 | 47°29'35 | 42 (6x) | 1747 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | AGGN | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Austria | Vorarlberg | Aggenstein | 010°33'27 | 47°32'14 | | 42 (6x) | 1987 |
| 45* | DOMI | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | Italy | Lombardia | Passo di Croce Domini | 010°24'33 | 45°54'27 | 14 (2x) | | 1880 |
| 46 | BALD | 5 | *Bupleurum ranunculoides* subsp. cf. *caricinum* (DC.) Arcangeli | Italy | Lombardia | Monte Baldo | 010°50'10 | 45°42'50 | 14 (2x) | | 2000 |
| 47 | KOPF | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Germany | Berchtesgadener Land, Bayern | Achenkopf, au pied de la croix | 012°57'11 | 47°41'60 | | 28 (4x) | 1568 |
| 48* | AMP | 5 | *Bupleurum ranunculoides* subsp. *caricinum* (DC.) Arcangeli | Italy | Udine | Ampezzo | 012°45'55 | 46°25'14 | 14 (2x) | | 1265 |
| 49 | AMAR | 5 | *Bupleurum ranunculoides* subsp. *caricinum* (DC.) Arcangeli | Italy | Udine | Col au-dessus d'Amaro | 013°04'16 | 46°23'33 | 14 (2x) | | 1082 |
| 50* | UGO | 5 | *Bupleurum ranunculoides* subsp. *caricinum* (DC.) Arcangeli | Italy | Udine | Ugovizza, Val Canale | 013°28'38 | 46°30'41 | 14 (2x) | | 862 |
| 51* | PLESA | 5 | *Bupleurum ranunculoides* subsp. cf. *ranunculoides* L. | Slovenia | Kras-Brkini, Slovenia | Gora Plesa | 014°03'04 | 45°46'18 | | 28 (4x) | 1262 |
| 52 | ZEIK | 5 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Austria | Steiermark | Zeiritzkampel | 014°43'14 | 47°29'22 | | 28 (4x) | 2049 |
| 53* | TATRA | 3 | *Bupleurum ranunculoides* subsp. *ranunculoides* L. | Poland | Tatra | Les Tatras | 20°00'20 | 49°09'51 | 14 (2x) | | 2000 |
| **Outgroup** | | | | | | | | | | | |
| 54 | FALCA | 2 | *Bupleurum falcatum* L. | Switzerland | Vaud | Aiguilles de Baulme | 006°28'08 | 46°47'26 | 16 (2x) | | 1485 |
| 55 | CERNU | 2 | *Bupleurum falcatum* subsp. *cernuum* Arcangeli | Slovenia | Kras-Brkini, Slovenia | Gora Kucelj | 013°50'58 | 45°55'42 | 16 (2x) | | 1211 |
| 56 | PETRE | 2 | *Bupleurum petraeum* L. | Italy | Como | Grigna Meridionale | 009°23'27 | 45°55'16 | 14 (2x) | | 2135 |
| 57 | ANGULO | 2 | *Bupleurum angulosum* L. | France | Hautes-Pyrénées | Entre le col Soulor et le col Aubisque | 000°17'40 | 42°57'54 | 14 (2x) | | 1365 |
| 58 | LONGI | 3 | *Bupleurum longifolium* L. | Switzerland | Neuchâtel | Creux du Van | 006°43'13 | 46°56'13 | 16 (2x) | | 1405 |




## Bayesian inference clustering analysis

AFLP scoring using the software Genographer yielded a total of 89 scorable loci when the two primers were combined (EACC-MCTG = 42 fragments and EACA-MCTA = 47). Results of the Bayesian inference clustering based on AFLP data set showed the best likelihood value at $K = 6$ (ln = -11613.4). Outgroup individuals were grouped all together in a single cluster (not illustrated). The five clusters within *Bupleurum ranunculoides* s.l. were geographically segregated. Whereas two thirds of the analysed populations were homogeneously assigned to a given cluster, 17 populations were assigned to a cluster with an assignment probability lower than 85 % (figure 3). The first cluster encompasses populations from the Oromediterranean part of France and Pyrenees (France cluster). The second cluster is composed of populations from the South-Eastern Alps (South-Eastern Alps cluster), whereas the third cluster is restricted to the Southern Alps (Southern Alps cluster). Finally, two non-geographically-segregated clusters were highlighted in the Northern Alps (Northern Alps 1 and 2 clusters). Many populations from the Central Alps were partially assigned to both Northern Alps and Southern Alps clusters. Similarly, two populations from the France cluster (1 PORTE and 3 CBIEL) and one from the South-Eastern cluster (51 PLESA) displayed strong admixture patterns (figure 3).

## Principal coordinate analyses (PCoA)

The first two axes of the PCoA explained 20 % of the genetic variance (figure 4A, B). The PCoA shows a main division along the first axis that separates all the diploids from other cytotypes except individuals from the Tatras. The left-right division corresponds to a split between individuals from the Northern Alps and Tatras with individuals from the Southern Alps and Oromediterranean part of France. According to the second axis, diploids are distributed into two distinct groups, whereas tetraploids and hexaploids are all grouped together (figure 4A). When looking at the distribution of the Bayesian inference clustering, the South-Eastern Alps and Southern Alps clusters are closely related; the same holds true for the Northern Alps 1 and 2 clusters.

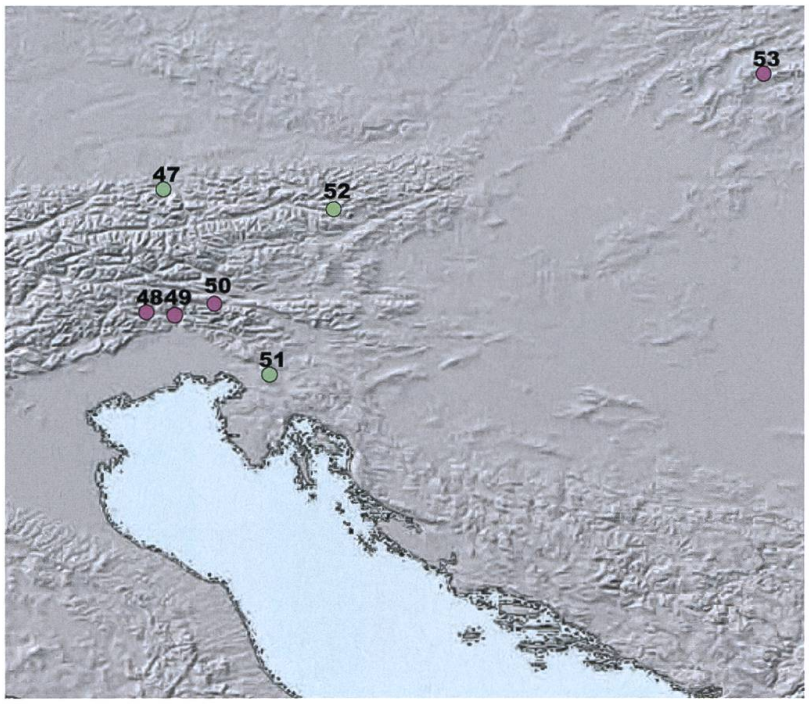



Figure 2. Geographic distribution of sampled populations of *Bupleurum ranunculoides* s.l. L., encompassing the major part of the species range. Numbers = population ID according to table 1. Populations without number correspond to literature references according to Küpfer (1974). Coloured pies represent ploidy level of the population. Purple refers to the diploids (2n = 2x = 14), light green refers to the tetraploid (2n = 2x = 28) and light blue are the hexaploids (2n = 6x = 42).

### Phylogenetic reconstruction

The phylogeny based on the Nei-Li distance applied to the AFLP dataset highlights a split between the Northern Alps and Tatras individuals from those from the Southern Alps, South of France and Pyrenees, roughly corresponding to a separation between populations from the Southern Alps (South-Eastern Alps, Southern Alps and France) and the Northern Alps (Northern Alps 1 and 2 clusters; figure 5) clusters. In more details, the Southern Alps encompasses four genetic clusters: one comprising the Oromediterranean part of France and the Pyrenees, the second comprising only the population 51 PLESA, the third including the three populations from the South-Eastern Alps and the fourth composed by Southern Alps individuals including individuals from Ticino and Lombardia. The Northern Alps cluster is divided into two sub-groups that match the two clusters previously highlighted with the STRUCTURE analysis (figure 3).

The ITS phylogeny reveals the same deep split between the Northern and Southern Alps, segregated by a mutation on position 61 of the sequenced amplicon (figure 6). Additional point mutations are present in several populations and a 6-mer deletion is found in three populations from the South-Eastern Alps cluster. One should note that one population, 51 PLESA, is characterized by a fixed polymorphism of the variation found on position 61.

## Discussion

### Phylogenetic relationships, genetic clustering and glacial refugia

By clustering all outgroups into one single genetic cluster while identifying five well-differentiated genetic clusters at the intra-specific scale, the Bayesian clustering analysis based on the AFLP dataset confirmed the suggested monophyly of the group *Bupleurum ranunculoides* s.l. (figures 2 and 4). A main dichotomy splits the species into a Southern group and a Northern group, as shown in the Nei-Li distance tree (figure 5), and corroborated by the ITS analysis

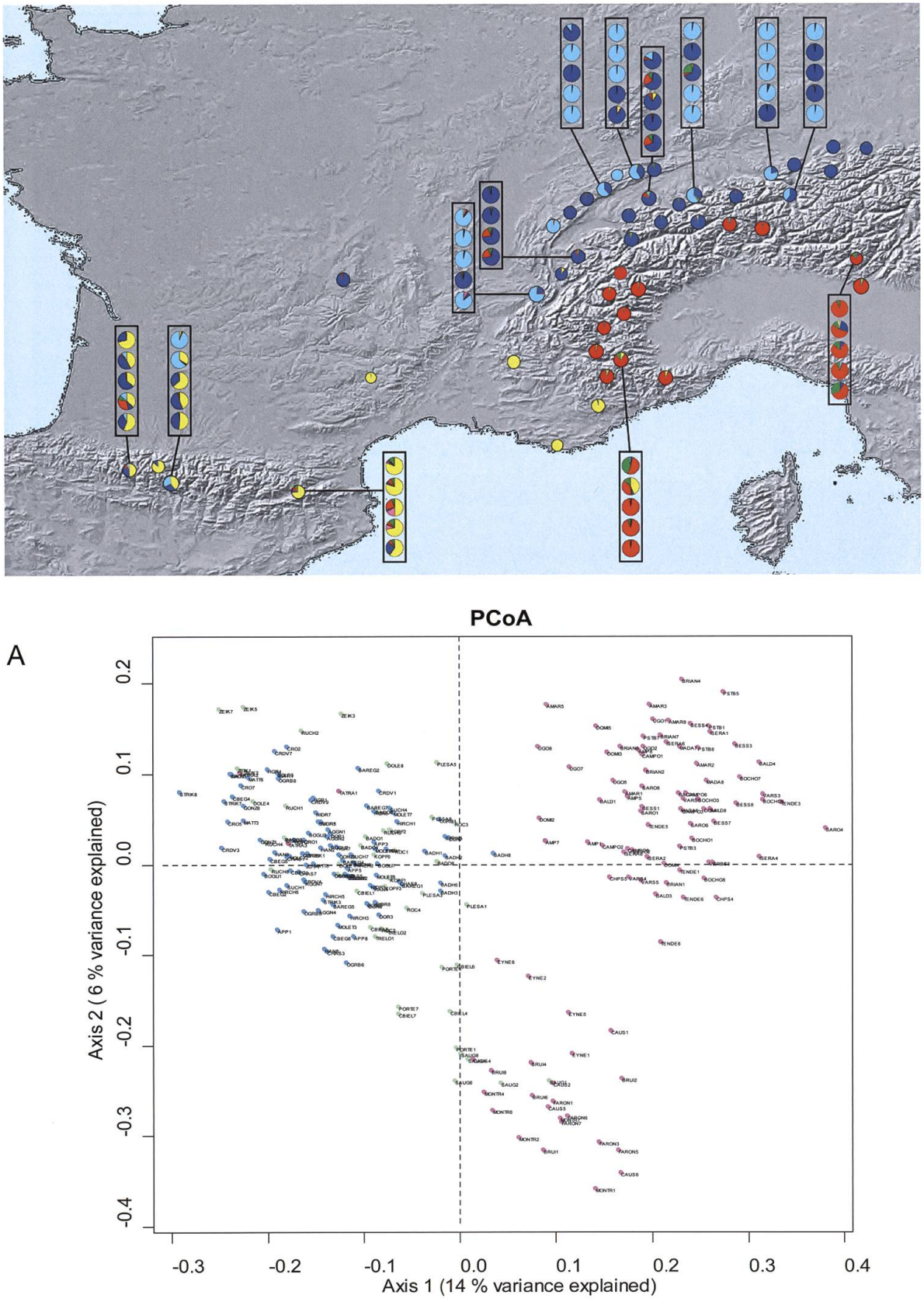



Figure 4. A) Principal coordinates analysis (PCoA) of *Bupleurum ranunculoides* s.l. AFLP data set based on Jaccard's similarity coefficient. The first axis explains 14 % of the observed variance and the second 6 %. Coloured variants present ploidy level according to figure 2 (purple = diploid, light green = tetraploid and light blue = hexaploid).

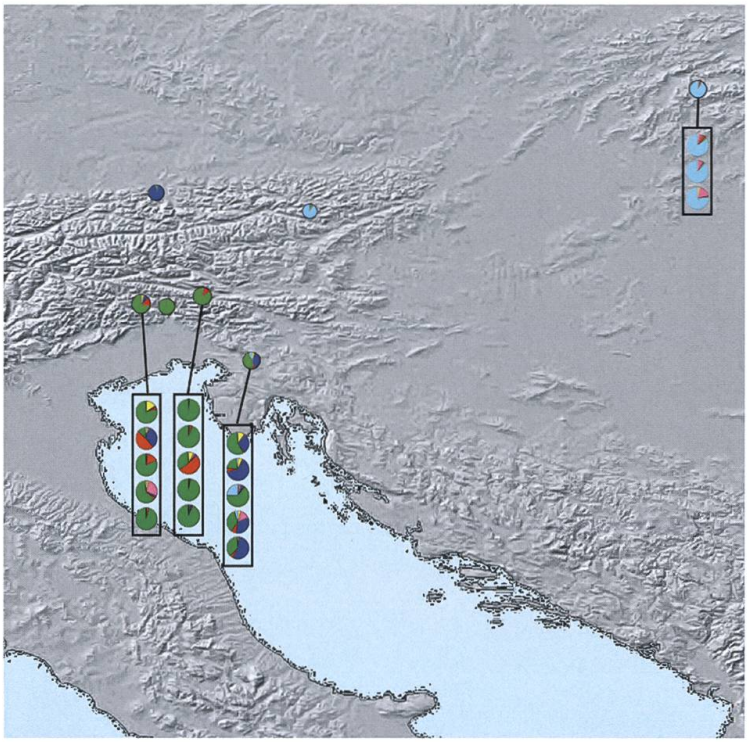


Figure 3. Genetic structuring of sampled populations of *Bupleurum ranunculoides* s.l. L. The five colours represent genetic clusters detected in the Bayesian clustering analyses of populations (STRUCTURE) of the AFLP data set. The sixth group consists only in outgroup species and is not mapped. Coloured variants within a pie present additional clustering detected. Individuals from populations with less than 85 % of their genetic pool assigned to only one cluster are represented one by one in a close-up rectangle. The size of the pies corresponds to the index of genetic diversity (low index is represented by small pie). Blue is for the Northern Alps 1 cluster, turquoise for Northern Alps 2 cluster, red for Southern Alps cluster, green for South-Eastern Alps cluster and yellow for the France cluster.

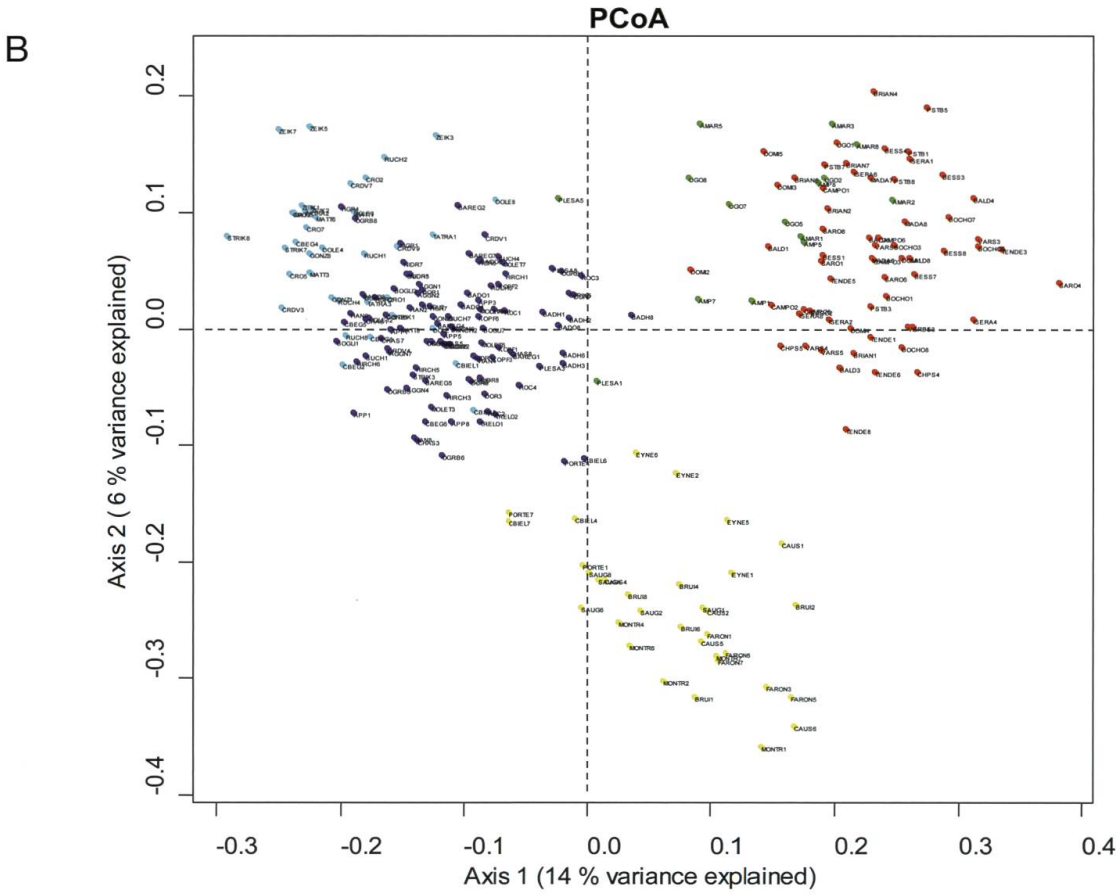



Figure 4. B) Same PCoA as in A) but with coloured variants representing genetic clusters detected in the Bayesian clustering analyses of populations as depicted in figure 3.

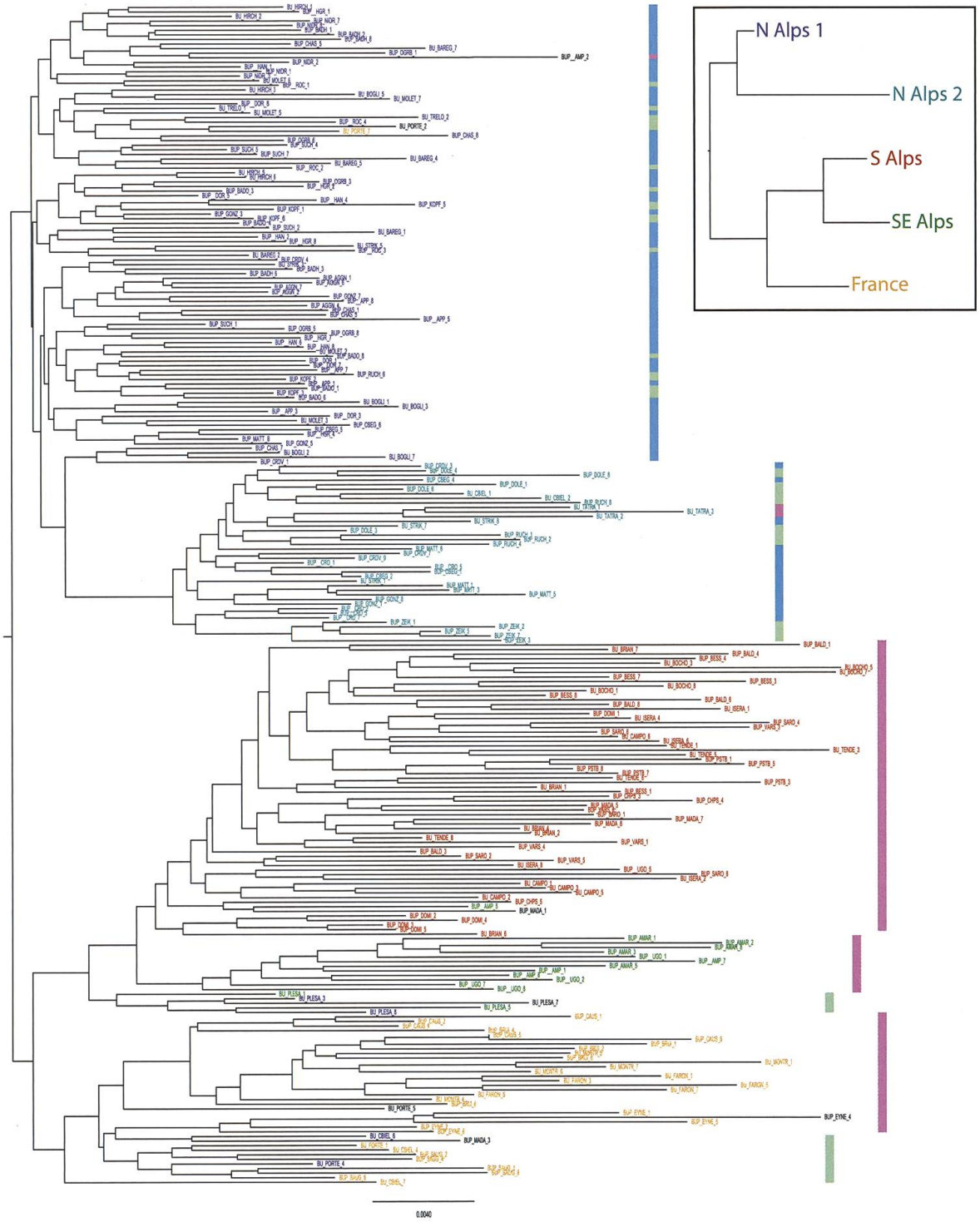


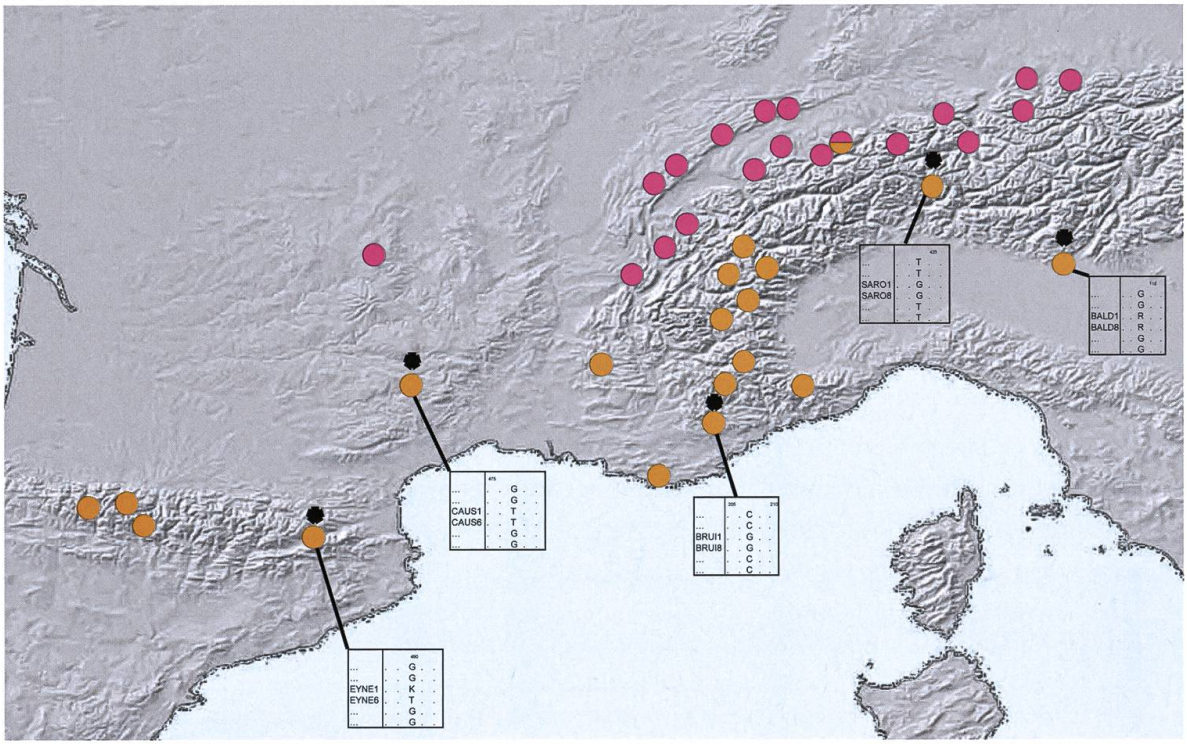

Figure 5. Neighbour-joining mid-point tree based on Nei-Li genetic distance among 257 individuals of *Bupleurum ranunculoides* based on the AFLP dataset. Colours on names correspond to the Bayesian clustering results as in figure 3 (blue is for the Northern Alps 1 cluster, turquoise for Northern Alps 2 cluster, red for Southern Alps cluster, green for South-Eastern Alps cluster, yellow for the France cluster and black for individuals not assigned to any cluster) and the ploidy level is illustrated by a line juxtaposed to the name on the right side of the tree (purple = 2x, light green = 4x and light blue = 6x). The tree is rooted according to the mid-point rooting method. The simplified phylogenetic tree on the top-right corner represents a neighbour joining cluster mid-point tree based on distances among clusters computed by the software STRUCTURE.

(figure 6). Strong segregation and reproductive isolation seem to have occurred between the two geographic regions, as corroborated by the difference in ploidy level between the Southern and Northern populations (figure 2). Three genetic pools are distributed in the Southern part of the Alps with a West to East repartition (respectively the France, South Alps and South-Eastern Alps clusters) and two genetic pools are widespread in the Northern part of the Alps without any clear geographic repartition (Northern Alps 1 and 2 clusters). Roughly, the five Bayesian clusters can be associated with the described subspecies as follows: *Bupleurum ranunculoides* subsp. *ranunculoides* L. with Northern Alps 1 and 2, *Bupleurum ranunculoides* subsp. *caricinum* (DC.) Arcangeli with the South-Eastern Alps and Southern Alps clusters, and *Bupleurum ranunculoides* subsp. *telonense* (Gren. ex Timb.-Lagr.) Bonnier with the France cluster. However, there are many exceptions to this association.

The France cluster includes both diploid and tetraploid populations found in the Oromediterranean part of France and the Pyrenees. Diploid populations harbour a private morphotype, distinct from the other *B. ranunculoides* and were determined in the field as the subspecies *B. r. telonense*. This morphotype develops at a lower mean altitude than the rest of the species and is also present in the Southern part of the Pyrenees. It is likely that these populations survived the Pleistocene glaciations *in situ* because of their non-glaciated habitat, thus favouring their morphological differentiation from other populations (Hewitt 2000). Contrary to the diploid populations, which show a low level of admixture, the three tetraploid populations display high levels of admixture with other clusters, and more specifically with both Northern Alps clusters. They are all situated in the Pyrenees, a previously demonstrated contact-zone for a large number of biota (Hewitt 2001). Thus, the admixture between different lineages could have promoted segmental allopolyploidization. Another hypothesis explaining this pattern could be the colonization by a tetraploid individual from the France cluster formed by homozygous autopolyploidy, which would have hybridized with an individual from one of the Northern Alps cluster. In both cases, the early formed tetraploid might have colonized the Western Pyrenees because of higher physiological and ecological flexibilities (Stebbins 1984).

The Southern Alps cluster is only composed of diploid populations. Their morphotypes are not uniform and vary along a gradient between the subspecies *caricinum* and *ranunculoides* (see table 1). This cluster shows an abrupt geographical differentiation with the France cluster, with only very limited introgression between them, a separation likely due to the Isere river acting as a barrier. Moreover, the strong differentiation with both Northern clusters might be due to the barrier formed by siliceous bedrocks distributed across the central Alps, which are not suitable for the species. Their Northern dispersal might have also been limited by the presence of Northern populations already occupying suitable habitats. This strong distinction with other clusters might be a consequence of *in situ* survival of the populations during the LGM, which was possible since the region remained free of ice and characterized by suitable calcareous habitats (Schönswetter & Tribsch 2005). Moreover, the specific ecological requirements of the species and/or mountains isolation may explain why only the Southern Alps cluster has colonized wider areas after glacier retreat, compared to the France cluster populations, which are restricted to isolated mountains and to the South-Eastern cluster populations that are surrounded by siliceous bedrocks.

The South-Eastern Alps cluster is composed of three diploid populations and a single tetraploid population, which were previously described as the subsp. *caricinum* due to their dif-




ferent morphotype and their lower mean altitude. Their divergence with the Southern Alps cluster could be a consequence of climate oscillations and survival of each cluster into their own refugia. Indeed, it was previously suggested that the populations from the South-Eastern Alps cluster had survived the Pleistocene glaciations *in situ* (Naciri 2007), demonstrating that the South-Eastern Alps may have acted as a glacial refugium for alpine plants growing on calcareous bedrocks. The 6-mer deletion found in the ITS region found in three populations out of four in the south-eastern tip of the Alps could confirm a long-lasting isolation. As for the France cluster, only the tetraploid population is showing a high level of admixture, and is also situated in a known contact zone (Ozenda 1985). For the latter, the fixed polymorphism at position 61 of the ITS region might reveal that population 51 PLESA was produced by an allopolyploid event following a cross between the former diploid lineage from the Northern Alps (yet only still present in the Tatras) and the diploid lineage from the South-Eastern Alps.

Both Northern clusters do not show any spatial structure, in contrast to the pattern observed for the three other clusters (see figure 3). They are distributed throughout the Northern edge of the geographic range of the species, from Central Massif to the Tatras. Interestingly, there is almost no sign of admixture between the two clusters. An examination of the morphotypes did not reveal any dissimilarity between the two clusters; all individual phenotypes were similar, independently of their ploidy level, and were determined as the subspecies *ranunculoides*. Contrary to the other clusters, they are composed of populations displaying the three different ploidy levels. In both clusters, hexaploid populations are surrounded by tetraploid populations. An hypothesis explaining this pattern might be that an ancestral vigorous tetraploid cytotype covered a huge area in the past, and was subsequently supplanted by hexaploid individuals in the central Alps. Many of the populations from the Jura and the Northern Alps are geographically close to areas supposed to have remained unglaciated during the LGM (which are thought to be peripheral refugia; Stehlik 2000; Stehlik *et al. 2002*; Tribsch

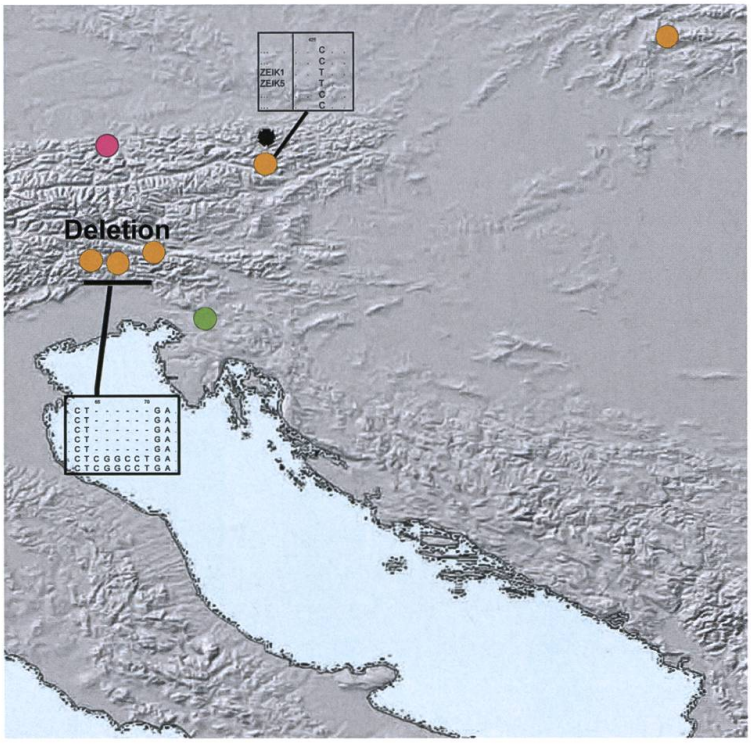



Figure 6. Geographical illustration of the results from the ITS region. The colours of the pies represent a single nucleotide variation on the 61st bp site (orange = G, green = A, and pink represents both A+G). One population 34STRIK had one individual with only G and the second with both A+G. Populations with a dot (•) showed a single nucleotide mutation on different bp sites. They are illustrated in a reported close up rectangle. At last, the 6 bp deletion present in five of the six individuals of South-Eastern Alps is illustrated in detail.

2002). According to these potential LGM refugia and the spatial aggregation of central hexaploid populations surrounded by tetraploid population, it is possible that the tetraploid cytotypes were distributed in a broad East to West range before the LGM and that they survived this last glacial period *in situ*.

Overall, our results suggest that the three genetic clusters from the Southern Alps, (*i. e.* France, South Alps and South-Eastern Alps clusters) have survived glaciations in Iberia, Italy, and Balkan refugia. Regarding the Northern Alps 1 and 2 clusters, our data do favour a hypothesis of *in situ* survival in a peripheral refugium from the Northern edge of the Alps *sensu lato* (including the Jura mountains, the Central Massif and the Tatra). Interestingly, the process of polyploidization has occurred in parallel in the lineages from both the Southern and Northern Alps (which are segregated basally in the intra-specific phylogeny; figures 5 and 6), producing tetraploid lineages in both, but hexaploid lineages only in the Northern Alps. One should note that admixture levels retrieved in several populations in our dataset show that despite genetic clusters are spatially segregated, some dispersal is at work in areas of secondary contact.

## Evolution of polyploidy

Diploid populations are found in all five genetic clusters addressed. Diploid populations are shown to be spread in the Southern Alps (and also in the Tatras), whereas hexaploid populations are only found in the Northern Alps, with tetraploid populations showing an intermediate distribution.

Our results suggest that a minimum of four events of polyploidization occurred in the sampled populations. The Slovenian population is supposed to be a segmental allopolyploidy between two different cytotypes. A second event occurred in the Pyrenees, and the two others (or more) occurred in the two Northern Alps clusters. The distribution of tetraploids on each side of the hexaploid range assumes that they were disseminated through the whole Alps and have been

probably supplanted by hexaploids through competitive processes. Such polyphyletic events of polyploidy from genetically differentiated lineages for both tetraploid and hexaploid cytotypes illustrate a dynamic system of divergence and polyploidization in the *B. ranunculoides* complex.

## Conclusion

As a general trend, this study on *Bupleurum ranunculoides* s.l. demonstrates that the different morphotypes exist as diploids and do not result of polyploidization. For the first time, tetraploid populations from the Eastern Alps have been described. The Eastern part of the species distribution seems quite intriguing since each of the three sampled tetraploid populations show high levels of admixture. This illustrates the low taxonomical value of the ploidy level in identifying subspecific entities of *B. ranunculoides*. Although the phylogeography of *B. ranunculoides* is not completely disentangled, our molecular analysis proposes new scenarios, involving in particular the different calcareous peripheral refugia surrounding the Alps. In contrast to previous cytological analyses (Cauwet 1970; Küpfer 1974; Cauwet 1979), it provides some evidence of a migration towards the South, from the Northern Alps and the Jura to the Pyrenees and to the Slovenian Dinaric Alps.

The ploidy analysis and molecular phylogeography of *Bupleurum ranunculoides* s.l. also presents new results about orophytic calcicolous species, by illustrating an emerging theory proposing that the major driver of spatial-genetic-structure in alpine plants is the soil ecology of a given species. Therefore, adequate substrate in refugia and along migration routes largely determines glacial survival and recolonization pathways in Alpine plants (Alvarez *et al.* 2009). Indeed, in this study, central siliceous bedrocks could have blocked Northern colonization of Southern clusters, whereas the edge of the Northern calcareous Alps might have acted as peripheral refugia. Moreover, during each ice age, the central Alps were deeply covered by glaciers that strengthened that barrier to migration. Thus, the central Alpine barrier may have been one of the predominant factor causing the South-North pseudo-vicariance highlighted by Küpfer (1974). In conclusion, the polyploid complex of *Bupleurum ranunculoides* s.l. is an interesting case study to investigate consequence of Pleistocene climatic oscillations on the polyploidy, intraspecific phylogeography and diversification of Alpine species, because of its dynamic evolution and long-lasting history.

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